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In Situ Detection of Hepatitis C Virus RNA in Liver Tissue Using a Digoxigenin-Labeled Probe Created During a Polymerase Chain Reaction

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The cellular localization of hepatitis C virus (HCV) RNA in liver tissue was studied by nonisotopic in situ hybridization using a digoxigenin-labeled cDNA probe created during a polymerase chain reaction on samples from 16 patients with chronic HCV infection. Hybridization signals were recognized in the cytoplasm of the hepatocytes, and a few hepatocytes had hybridization signals in the nucleus as well. HCV RNA positive hepatocytes were found in 1 of 9 patients with chronic persistent hepatitis, 2 of 5 patients with chronic active hepatitis, and in each of 2 patients with chronic active hepatitis and cirrhosis. Positive signals were found in many hepatocytes within the lobule in liver sections of patients with advanced chronic active hepatitis. A number of HCV RNA positive hepatocytes were found in nodules, but not in the area of fibrosis. On the other hand, positive signals were found in a few hepatocytes scattered in the lobule in a patient with chronic persistent hepatitis. The mean ALT levels in the patients with positive signal (175.6 ± 44.2 U/L) were significantly higher than in those without a signal (70.27 ± 16.1 U/L) ($P < 0.05$). The findings suggest that a larger amount of HCV may be present during the advanced than during the early stages of type C hepatitis and nonisotopic in situ hybridization using a digoxigenin-labeled HCV cDNA probe created during a polymerase chain reaction deserves wider application for the detection of HCV replication in specimens.

KEY WORDS: chronic hepatitis C, in situ hybridization, digoxigenin labeling, polymerase chain reaction

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of non-A, non-B hepatitis that is associated with the development of cirrhosis and hepatocellular carcinoma [Choo et al., 1989]. Sensitive immunoassays which detect antibody to HCV (anti-HCV) are generally used to diagnose HCV infection [Kuo et al., 1989]. HCV RNA can be detected in serum and liver of infective individuals using the reverse transcription-polymerase chain reaction (RT-PCR) [Garson et al., 1990]. However, PCR of HCV RNA extracted from either serum or liver tissue cannot provide information regarding the site of infected cells and the relationship between the existence of the infected cells and the pathological features of liver specimens. In situ detection of HCV RNA at the single-cell level should be helpful in gaining insights into the pathogenesis of HCV-induced liver damage and assessing the status of HCV replication in specimens.

PCR has been used to synthesize cDNA probes and a nonisotopic cDNA probe produced during amplification has been used to confirm the presence or absence of specific sequences following amplification of genomic DNA [Dennis et al., 1988]. This method was used successfully to confirm the HCV specificity of amplified fragments [Geiger and Caselmann, 1992].

In this study, a rapid and simple in situ hybridization technique is described for detecting HCV RNA in liver sections with a digoxigenin-labeled cDNA probe created during PCR. The histopathological features of HCV infected specimens were identified in patients with chronic HCV infection.

MATERIALS AND METHODS

Patients

Liver specimens were obtained by needle biopsy from 16 patients with chronic liver disease who were seropositive for anti-HCV and HCV RNA detected by second generation enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, Chicago, IL) and a RT-PCR, respectively. All patients except one were seronega-

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tive for HBsAg. The 10 men and six women ranged in age from 24 to 66 years (mean 45.2 years). Their serum alanine aminotransferase (ALT) levels ranged from 12 to 284 IU/L (mean 103.5 IU/L). Nine patients had histological findings of chronic persistent hepatitis (CPH), five of chronic active hepatitis (CAH) and two of CAH with cirrhosis. A part of each liver specimen was used for diagnostic purposes. The remainder (10-mm long specimens) was embedded in OCT compound (Tissue Tek; Metpath, Tegerboro, NJ), immediately snap frozen in liquid nitrogen, and stored at −70°C.

Tissue Preparation for In Situ Hybridization

The frozen specimens were cut at 5–7 μm on cryostat, and individual sections were fixed with a solution of freshly prepared 4% paraformaldehyde in phosphate-buffered saline at 4°C for 1 min. The slides were then stored in 70% ethanol at 4°C until use.

Nested PCR

RT-PCR was used to detect HCV RNA in serum samples. The details of the method have been described previously [Inchauspe et al., 1991]. In brief, 100 μl of serum was used for the extraction of RNA according to the method of Chomczynski and Sacchi [1987]. Twenty microliters of serum equivalent of RNA mixture was employed for RT-PCR using external primer 20 (5′-GAATCTGTGCTCATGGTGCA-3′, antisense, position from −6 to 13) and 19 (5′-GCGACACTCCACCATAGAT, sense, position from −324 to −304) located in the 5′-untranslated region of the HCV genome. A second-round of amplification was performed using a 10 μl aliquot of the first-round PCR product and internal primer 22 (5′-ACTCGCAAGCACCCTATCA-3′, antisense, position from −48 to −32) and 21 (5′-CTGTGAGGAACTACTGTCT-3′, sense, position from −301 to −281). The second-round PCR products were subjected to electrophoresis in 1.4% agarose with ethidium bromide. A signal of the expected size of 269 bps was visualized.

A second-round PCR was carried out using 10 μl of first round PCR product from serum to generate digoxigenin-labeled HCV cDNA probe. Digoxigenin-linked deoxynucleotide triphosphates (Boehringer Mannheim, Germany) (molar ratio dTTP/DIG-dUTP = 5:1) were incorporated into the newly synthesized cDNA strands during the second-round of amplification with Taq polymerase (Promega, USA) [Geiger et al., 1992]. The production of digoxigenin-labeled PCR product was confirmed by a slightly decreased electrophoretic mobility on agarose when compared with the unlabeled PCR product (Fig. 1). The resulting PCR product was purified by ethanol precipitation. The pellet was dissolved with 100 μl of 10 mM Tris-HCL, 1 mM EDTA (pH 8.0) and used for in situ hybridization [Dennis et al., 1988]. The labeled PCR product from serum of a patient was used as a probe to detect HCV RNA in every liver tissue studied. The patient whose serum was used to generate labeled probe was a 46-year-old man. He had anti-HCV, but not HBsAg. Chronic active hepatitis was diagnosed histopathologically. Anti-HCV remained positive, and

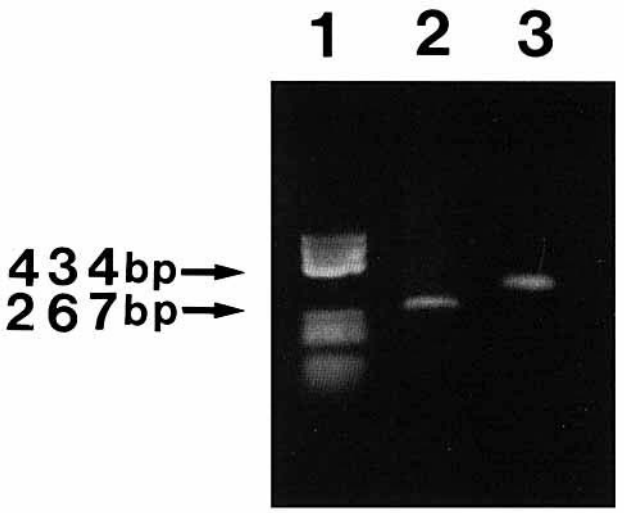

Fig. 1. Production of a 269-bp digoxigenin-labeled HCV cDNA probe using PCR. PCR products were analyzed after fractionation on a 1.4% agarose gel and revealed after ethidium bromide staining. **Lane 1,** molecular weight marker pBR322 DNA/Hae III digest; **lane 2,** unlabeled PCR product; **lane 3,** digoxigenin-labeled PCR product.

ALT levels fluctuated at 50–250 IU/L during a period of 18 months. HCV infection was confirmed by detecting HCV RNA in serum.

In Situ Hybridization

The stored sections were washed in distilled water and treated for 15 min at 37°C with proteinase K (5 μg/ml, Boehringer Mannheim, Germany) followed by two washings in distilled water [Haase et al., 1984]. This is a critical step. We tried initially different concentration of proteinase K 2.5, 5, and 10 μg/ml for 15 min. Most samples were processed satisfactorily with proteinase K 5 μg/ml and were dehydrated through a graded series of ethanols (70–100%). The sections were prehybridized in hybridization cocktail (Amresco; Solon, Ohio) containing yeast transfer RNA (250 mg/ml) for 3 hr at 37°C, washed with 1 × SSC and then overlaid with 30 μl of hybridization solution containing 1 μl of the denatured digoxigenin-labeled HCV probe. After 12 to 16 hr of incubation at 37°C, the digoxigenin-labeled hybrids were detected with a digoxigenin antibody/alkaline phosphatase conjugate and an enzyme substrate/chromogen (NBT/BCIP) according to the manufacturer's instructions (Boehringer Mannheim, Germany). In the original experiments, we treated slides with ethanol and xylene, resulting in the development of dark blue hybridization signals. But hybridization signals were removed or weakened after ethanol and xylene treatment in the tissue with weak signals due to solubility of NBT/BCIP reaction product in organic solvents such as ethanol and xylene. In subsequent work, carried out without ethanol and xylene, the hybridization signals became brownish blue.

To confirm the specificity of the hybridization, several controls were carried out in the serial sections. The dehydrated sections which were processed through a graded series of ethanol were digested with 25 μg/ml ribo-

nuclease A and 40 U/ml ribonuclease T1 (Boehringer Mannheim, Germany) at 37°C for 30 min, then washed in 0.1% diethyl pyrocarbonate (Sigma) at room temperature for 5 min. The sections were washed in 2 × SSC at room temperature for 5 min and dehydrated with ethanol. The dehydrated sections were processed for hybridization. A cDNA probe specific for cytomegalovirus 272 nucleotides in length was synthesized during PCR, and used as a control [Stenberg et al., 1984]. Digoxigenin-labeled pBR 322 produced by random priming was also used instead of HCV probe. Prehybridization of sections with a large excess of unlabeled probe was undertaken to eliminate the hybridization signal. In absorption studies, the sections were prehybridized in hybridization cocktail containing a 30-fold excess of unlabeled HCV probe for 2 or 3 hr at 37°C, washed with 1 × SSC and then processed for hybridization. In some sections, hybridization was carried out in hybridization solution without the specific probe. The section of three liver specimens of a chronic HBsAg carrier and one of rat liver specimen were included in every experiment as negative controls. All samples were repeated at least twice.

Statistics

Statistical analysis was carried out using Student's t-test.

RESULTS

The specificity of the nonisotopic in situ hybridization (NISH) was examined subsequently in a number of control experiments. RNase treatment of liver sections before hybridization abolished the HCV signals (Fig. 2). Staining was not detected when tissue sections were hybridized with a digoxigenin-labeled cytomegalovirus DNA probe created during PCR and pBR322 labeled by random priming instead of the probe for HCV genome. Staining was also not detected when hybridization was carried out without the specific probe for HCV genome. In preabsorption studies an excess of unlabeled HCV probes that were applied during prehybridization step reduced the HCV signals by −80%. Three liver specimens of chronic HBsAg carrier and one of rat did not show hybridization signals.

HCV-specific RNA was recognized in hepatocytes of patients with chronic hepatitis (Figs. 2 and 3). Positive signals were observed in the cytoplasm of the hepatocytes, and a few hepatocytes had hybridization signals in the nucleus. HCV RNA was not stained in infiltrating lymphocytes in liver sections. HCV RNA-positive hepatocytes were found in 1 of 9 patients with CPH, 2 of 5 patients with CAH, and in each of 2 patients with CAH and liver cirrhosis, suggesting that there is a tendency toward increased levels of HCV replication according to the progression of the histopathological changes (Table I). With respect to the mean levels of serum ALT, a significant difference was seen between HCV-positive groups (176.6 ± 44.2 U/L) and HCV-negative groups (70.27 ± 16.1 U/L) ($P < 0.05$).

The positive signals were found in the scattered hepatocytes of all acini and in some hepatocytes in the area of necrosis. The staining patterns were divided into three types: diffuse, cluster or focal. The staining pattern was focal in one case of CPH. In two cases of CAH, the diffuse pattern was seen in one and the cluster pattern in the other one. In two cases of CAH and cirrhosis. The cluster pattern was seen in one and the diffuse pattern in the other one. A number of signal positive hepatocytes were found within regenerating nodules, but HCV RNA was not found in the area of fibrosis (Fig. 3).

DISCUSSION

Recently, some groups have undertaken in situ hybridization to detect HCV genome in liver sections [Lamas et al., 1992; Haruna et al., 1993; Nouri-Aria et al., 1993; Tsutsumi et al., 1994]. The use of NISH has become common because most of the nonradioactive methods are now as sensitive as autoradiography but they do not have the disadvantages of the isotopic methods, such as the long assay time and the requirement for special laboratory facilities. Using PCR, large quantities of biotinylated probes have been made, and used to confirm the specificity of amplified fragment by hybridization with this probe [Dennis et al., 1988]. NISH has been shown to be as sensitive as an isotopic procedure, and has been used successfully for the detection of hepatitis B virus [Naoumov et al., 1993]. Geiger et al. [1992] created a digoxigenin-labeled HCV probe by a reverse PCR of RNA extracted from the serum of a patient, and confirmed the HCV specificity of amplified fragments. We synthesized a digoxigenin-labeled HCV cDNA probe by a reverse PCR of RNA extracted from the serum of a patient with chronic type C hepatitis. After incorporation of digoxigenin-dUTP during the second round of nested PCR, this probe was used for the in situ detection of HCV RNA in liver sections. The labeled hybridization probe is stable for a considerable period of time. Using this technique, specific HCV signals were detected in hepatocytes within 2 days.

HCV RNA was found in 5 of 16 liver specimens by NISH. The number of the HCV RNA-positive cells had a tendency to increase according to the progression of the histopathological changes of the liver, which is consistent with the previous reports [Haruna et al., 1993; Tsutsumi et al., 1994]. A previous report on the quantification of HCV RNA showed a correlation between the relative quantities of serum HCV RNA and histopathological changes [Kato et al., 1993]. The results of these studies may support a direct cytopathic role of HCV in patients with high level viremia. In chronic type B hepatitis, there is an inverse relationship between the serum level of HBV DNA and the degree of liver damage [Chu et al., 1985]. In contrast, the studies using in situ hybridization showed that CAH is associated with an accumulation of cytoplasmic HBV DNA, while in patients with CPH the cytoplasmic expression of HBV DNA is weaker or not detectable [Naoumov et al., 1993; Burrell et al.,1984]. Sakamoto et al. [1994] found a weak correlation between the amount of liver HCV RNA and that of HCV RNA in plasma in small number of patients. In contrast to our observation, they reported that there

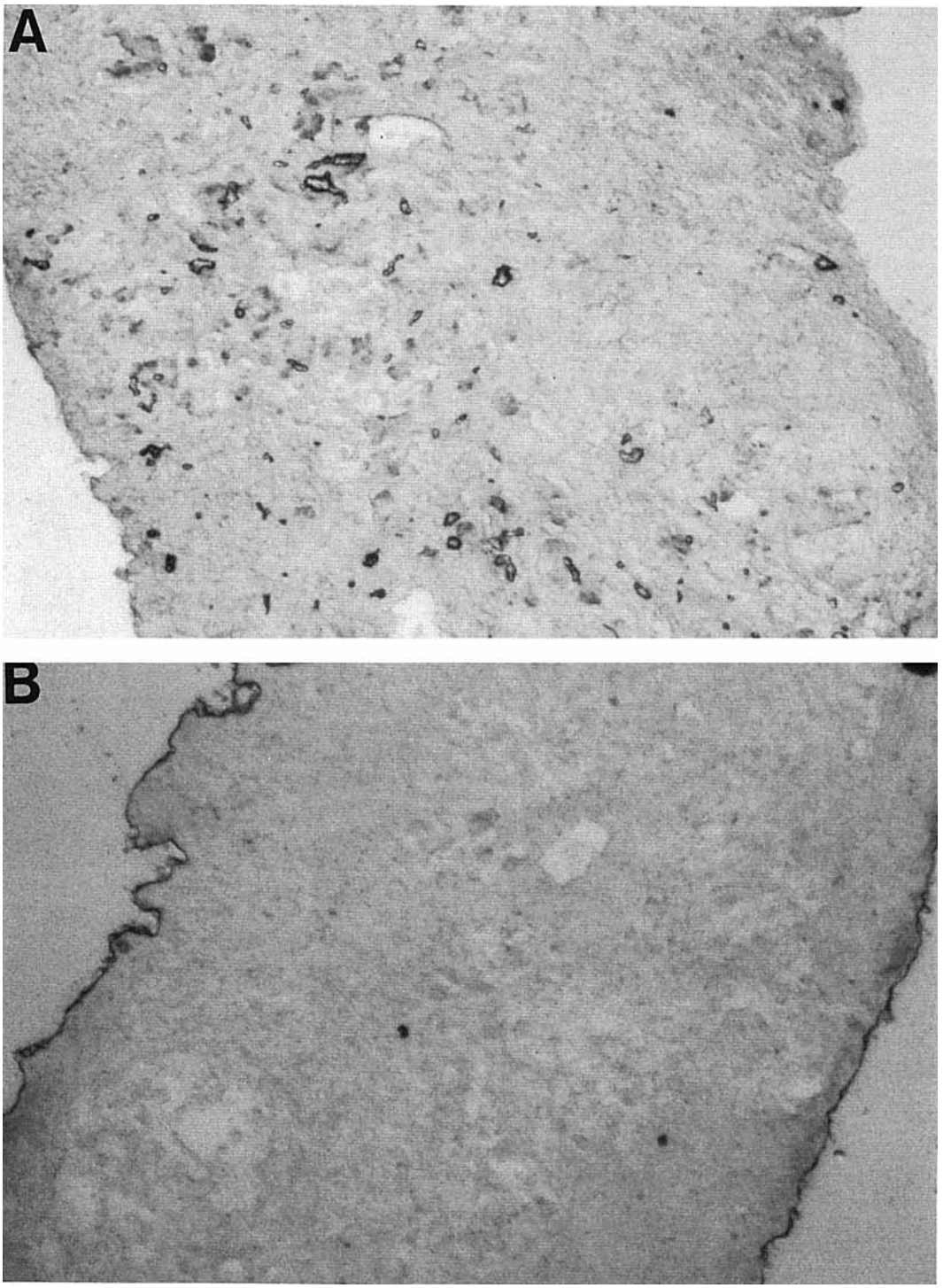

Fig. 2. NISH performed on liver tissue from a HCV-infected patient using a digoxigenin-labeled HCV cDNA probe. The HCV RNA was detected in scattered hepatocytes **(A).** Hybridization signals were abolished by pretreatment with RNase **(B).** (Original magnification ×40.)

was no correlation of histopathological findings with the amount of liver HCV RNA as detected by competitive RT-PCR. Nouri-Aria et al. [1993] also found a lack of correlation between the degree of hepatocellular damage and the prevalence of HCV RNA in liver tissue as detected by ISH. These discrepancies may be due to the small number of patients with CPH in their studies or different sensitivities of in situ hybridization techniques.

We observed positive signals in some hepatocytes at the area of piecemeal necrosis. The similar observation

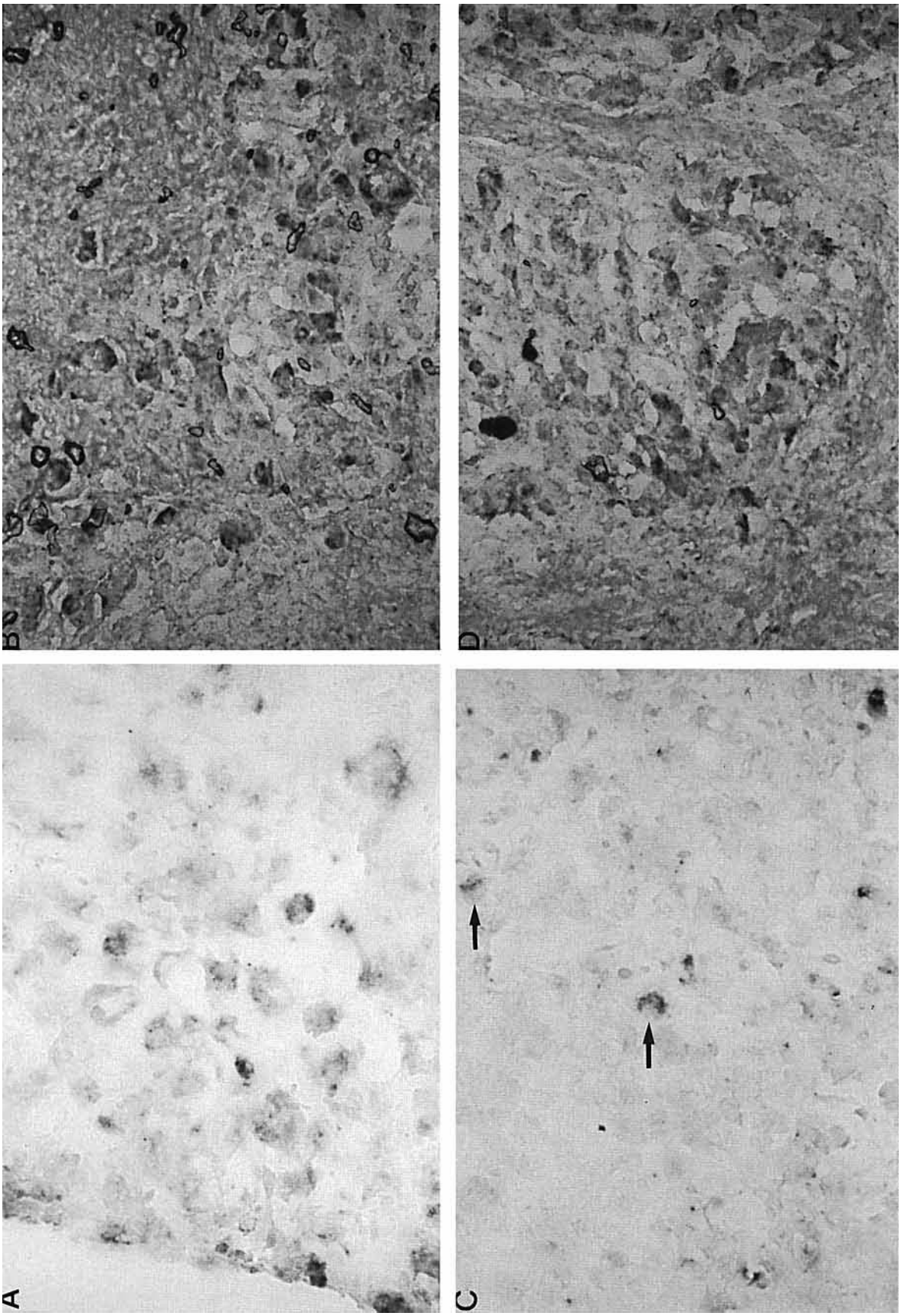

Fig. 3. Detection of HCV RNA in liver tissue from HCV infected patients by NISH. The different staining patterns of HCV RNA found in specimens were diffuse **(A)**, cluster **(B)**, and focal **(C)**. A number of hybridization signal positive hepatocytes were found within regenerating nodules **(D)**. The arrows indicate positive cells. (Original magnification ×200.)

TABLE I. Clinical Data and Results of In Situ Hybridization

No.	Age (yr)/sex	ALT (IU/L)	Liver histology	HBsAg	Liver	
					HCV RNA	Staining pattern
1.	34/F	21	CPH	–	–	–
2.	50/F	57	CPH	–	–	–
3.	31/M	165	CPH	–	–	–
4.	56/F	20	CPH	–	–	–
5.	57/F	29	CPH	–	–	–
6.	48/M	284	CPH	–	+	Focal
7.	63/F	45	CPH	–	–	–
8.	24/M	12	CPH	–	–	–
9.	35/M	79	CPH	–	–	–
10.	31/F	113	CAH	–	–	–
11.	66/M	124	CAH	–	–	–
12.	46/M	146	CAH	–	+	Diffuse
13.	38/M	108	CAH	–	–	–
14.	47/M	257	CAH	–	+	Cluster
15.	50/M	102	CAH/C	+	+	Cluster
16.	47/M	94	CAH/C	–	+	Diffuse

CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; C, cirrhosis.

was reported in chronic type B hepatitis [Michitaka et al., 1988], and HCV specific cytotoxic T lymphocytes were detected at the site of tissue damage in persons with chronic HCV infection [Koziel et al., 1992]. These experiments suggest that immune-mediated mechanisms may also play a role in the development of hepatocellular necrosis in chronic HCV infection.

The absence of HCV-positive hepatocyte in the majority of patients with CPH in this study is consistent with the view that HCV RNA in the serum or liver can be detected after amplification by PCR. However, many HCV-positive hepatocytes were found within regenerating nodules and scattered in the lobule from patients with advanced stage of chronic hepatitis, as shown for chronic type B hepatitis [Han et al., 1993]. This observation suggests a wide range of the levels of HCV replication in the different stages of chronic infection. The hybridization signals in the positive hepatocytes varied in intensity within the same tissue sections. Perhaps that was because individual hepatocytes may differ in the susceptibility to an HCV infection. A previous report showing no correlation between the number of hepatocytes containing HBcAg and the proportion of neighboring sinusoidal cells expressing interferon-α may support this hypothesis [Nouri-Aria et al., 1991], although the in situ localization of interferon in liver tissue of HCV infection is not yet known.

Tsutsumi et al. [1994] reported that HCV-NS5 antigen and HCV RNA in liver sections were positive in one of four patients who lost HCV RNA in serum after interferon therapy, and the detection of HCV RNA by in situ hybridization was more sensitive than HCV-NS5 antigen staining. Di Bisceglie et al. [1993] reported that the degree of HCV antigen staining decreased following interferon therapy, and HCV antigen became undetectable after therapy in those patients who had a long-term response to treatment. These reports suggest a possible role of HCV RNA in in situ hybridization in determining the effect of interferon and evaluating the changes of HCV replication during the course of HCV infection.

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